



Brewers' rice, a by-product from rice processing, has antiproliferative activity on human colorectal cancer (HT-29) cell line

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Abstract

Colon carcinogenesis is a malignant tumor, and is well-known as the third leading cancer, which contributes to high mortality and morbidity worldwide. Brewers' rice, known locally as *temukut*, consists of a mixture of broken kernels with rice bran and rice germ which is a by-product produced in the rice industry. Although extensive studies on the anti-cancer properties of rice bran, published data on the cytotoxicity of brewers' rice are very limited. The present study was conducted to evaluate the apoptosis induction capability of the water extract of brewers' rice (WBR) on human colorectal cancer (HT-29) cell line. The HT-29 cells were treated with various concentrations (16, 32, and 64 µg/mL) of WBR for 24 and 48 hours. The morphological analysis of apoptotic cells was evaluated using inverted light microscope and fluorescence microscope. The apoptotic HT-29 cells was evaluated using Annexin V-FITC and propidium iodide (PI) staining apoptosis test and cell cycle analyses. The data obtained were evaluated using a one-way analysis of variance (ANOVA) and $P < 0.05$ was considered statistical significant. Overall analyses indicated that WBR induced typical characteristics of apoptosis in HT-29 cells, including nuclear fragmentation (NF), nuclear compaction (NC), apoptotic bodies (AB), cellular shrinkage (CS), and chromatin condensation (CC), as visualized under inverted light microscope and fluorescence microscope. Cell cycle analyses and Annexin V-FITC and propidium iodide (PI) staining apoptosis test using flow cytometry revealed that WBR induced apoptotic population in HT-29 cells. In this study, our findings provide clear evidence that WBR inhibits the growth of HT-29 cells via induction of apoptosis. Taken together, we suggest that WBR may be a potential candidate for the prevention and treatment of colorectal cancer.

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Keywords

Water extract of brewers' rice

Colorectal cancer

Cell morphology

Apoptosis

Introduction

Cancer is one of the most common causes of death in both developed and developing countries and has become a significant public health concern worldwide (Jemal *et al.*, 2010). Most of the cancer cases and deaths worldwide are preventable (Tantamango-Bartley *et al.*, 2013). Colon carcinogenesis is a malignant tumor, and is well-known as the third leading cancer, which contributes to high mortality and morbidity worldwide (Haggar and Boushey, 2009). Several studies reported that natural products play a critical role against cancer (Kuno *et al.*, 2012; Shin *et al.*, 2012; Wu *et al.*, 2013). Anti-cancer drug therapies are usually toxic, immune-suppressive,

mutagenic, and even carcinogenic (McWhinney *et al.*, 2009; Minami *et al.*, 2010). Combination of two or more components in drug design has shown clinically synergistic benefits against the cancer treatment (Lao and Brenner, 2004). Thus, modification of the dietary determinants could delay the progression of cancers (Kannan *et al.*, 2009).

Rice (*Oryza sativa*), an essential cereal crop for half of the humanity, and has been widely demonstrated as a chemopreventive component (Henderson *et al.*, 2012). The dietary rice bran has been reported to contain many beneficial effects against different types of cancers, included leukemia, breast, lung, liver, cervical, stomach, and colorectal cancers (Henderson *et al.*, 2012; Chen *et al.*, 2012).

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Antioxidant compounds in rice bran have been demonstrated to contribute to these significant health benefits (Canan *et al.*, 2012). In addition to the anti-cancer property observed in rice bran, brewers' rice is another by-product in the rice industry that contained a significant nutritional value.

Brewers' rice, known locally as *temukut*, is a mixture of broken kernels with rice bran and rice germ which is usually removed during the rice milling process (Adilah *et al.*, 2014). The production of brewers' rice in rice industry is described in more detail in Esa *et al.* (2013). To date, very limited attention has been paid to the impact of water extract of brewers' rice (WBR) on cancer. Our preliminary study showed that WBR was cytotoxic against human colorectal cancer (HT-29) cells after incubated for 72 hours (Tan *et al.*, 2013, 2015). Therefore, we further evaluated whether the extract is also effective if the period of treatment is reduced to 24 and 48 hours.

Materials and Methods

Chemicals and reagents

Acridine orange (AO), propidium iodide (PI), phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA) (purified grade, approx. 99%), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Mycoplex™ fetal bovine serum (FBS), penicillin and streptomycin (100×), and trypsin EDTA (1×) were purchased from PAA Laboratories GmbH (Pasching, Austria). A Cycle TEST PLUS DNA Reagent Kit and Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). All other chemicals and reagents used were of analytical grade and bought from Sigma-Aldrich (St. Louis, MO, USA).

Brewers' rice

The brewers' rice sample (rice variety MR 219) was collected from BERNAS Milling Plant at Seri Tiram Jaya, Selangor, Malaysia. Stabilization of brewers' rice sample was performed followed the method as described in our earlier study (Tan *et al.*, 2013).

Preparation of extract from brewers' rice

The detailed procedures for extraction of brewers' rice using water as a solvent were described previously by Tan *et al.* (2013).

Cell culture

The colorectal cancer (HT-29) cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). HT-29 cells were grown in DMEM supplemented with 10% (v/v) of FBS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. HT-29 cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂ atmosphere.

Morphological analysis of apoptotic cells by inverted light microscope

The HT-29 cells were seeded at a density 1×10^6 cells/mL into each well of a 6-well plate and incubated for 24 hours. HT-29 cells were treated with various concentrations (16, 32, and 64 µg/mL) of WBR for 24 and 48 hours. The morphological changes of the HT-29 cells were viewed under an inverted light microscope (Olympus, Center Valley, PA, USA) at 200× magnification.

Morphological analysis of apoptotic cells by acridine orange (AO)-propidium iodide (PI) double staining

1×10^6 cells/mL of HT-29 cells were seeded into each well of a 6-well plate and incubated overnight at 37 °C in a humidified atmosphere with 5% CO₂ atmosphere. WBR was added into each well with different concentrations (16, 32, and 64 µg/mL) and incubated for 24 and 48 hours. After incubation, 10 µL (1 mg/mL) of AO and PI each, at 1:1 mixture was added into each well. The cells were observed under a fluorescence microscope (Olympus, Center Valley, PA, USA) at 400× magnification.

Annexin V-FITC and PI staining apoptosis test

The HT-29 cells were seeded at a density of 1×10^6 cells in a culture flask for 24 hours. The culture medium was replaced with fresh aliquots containing three different concentrations (16, 32, and 64 µg/mL) of WBR. The subsequent procedures were conducted following to the manufacturer's protocols of Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). After 24 and 48 hours exposure to WBR, cells at a density of 1×10^6 cells were trypsinized and rinsed twice with PBS-BSA-EDTA, and the cell pellet was re-suspended in 100 µL of 1× binding buffer (0.1 M Hepes/NaOH, pH 7.4; 1.4 M NaCl₂, 25 mM CaCl₂). Next, 5 µL of Annexin V-FITC and 10 µL of PI were added and incubated in the dark for 10 minutes. Subsequently, 400 µL of 1× binding buffer was added and the fluorescence of the cells was analyzed using flow cytometry FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cycle analysis by flow cytometer

The Cycle TEST PLUS DNA Reagent Kit (BD Biosciences) was used to detect cell cycle arrest, according to the manufacturer's instructions. The HT-29 cells were seeded at a density of 1×10^6 cells in a 25 cm² culture flask for 24 hours, followed by exposure to WBR at three different concentrations (16, 32, and 64 µg/mL). After 48 hours, the exposed cells were collected in the original medium. Cells were then pelleted by centrifugation at $300 \times g$ at room temperature for 5 minutes and washed with buffer solution. Cells were added with 250 µL of Solution A (trypsin buffer) and 200 µL of Solution B (trypsin inhibitor and RNase buffer) and allowed to react at room temperature for 10 minutes, respectively. Cold Solution C (200 µL of PI stain solution) was added and incubated for an additional 10 minutes in the dark on ice. Cells were filtered through a 35-µm cell strainer cap. Data acquisition and analyses were carried out using flow cytometry FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest 3.3 software.

Statistical analyses

Statistical analysis was conducted using the Statistical Package for Social Science (SPSS) version 19.0. Statistical data were expressed as the mean \pm standard deviation (mean \pm SD). The data obtained were evaluated using a one-way analysis of variance (ANOVA) and $p < 0.05$ was considered statistical significant.

Results and Discussion

Morphological evaluation of apoptotic cells by inverted light microscope

As mentioned earlier, the current study is an extension of our previous work, which demonstrated that water extract of brewers' rice (WBR) is cytotoxic against colorectal cancer (HT-29) cell line with IC₅₀ values of 38.33 ± 6.51 µg/mL and 21.88 ± 12.43 µg/mL, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, respectively after incubated for 72 hours (Tan *et al.*, 2013, 2015).

Any crude extract exerts potentially cytotoxic activities should have an IC₅₀ less than 100 µg/mL (Prayong *et al.*, 2008). These findings revealed that WBR is sensitive against HT-29 cells, indicating that WBR can inhibit the proliferation of HT-29 cells. Due to this result, we further evaluated whether the extract is also effective if the period of treatment is reduced to 24 and 48 hours. Therefore, the morphological changes, mode of cell death, apoptosis induction, and

cell cycle arrest in response to WBR against HT-29 cells were further analyzed. Furthermore, given a broad cytotoxicity range of WBR against HT-29 cell line determined by the MTT and LDH assays, only three concentrations (16, 32, and 64 µg/mL) were selected.

To obtain further insights into the growth inhibitory effects of WBR in HT-29 cells are associated with the apoptotic processes, the induction of apoptotic cell death by morphological and biochemical methods were investigated. The morphological changes of apoptosis include nuclear fragmentation, chromatin condensation, and cell shrinkage (Suzuki *et al.*, 1998). The HT-29 cells were incubated with WBR for 24 and 48 hours, and their morphological changes were evaluated through an inverted light microscope. As illustrated in Figure 1, the population of HT-29 cells reduced with increasing concentrations of WBR. However, the untreated HT-29 cells were distributed evenly on the substratum (Figure 1A). Prominent apoptotic morphological features such as nuclear fragmentation (NF), nuclear compaction (NC), apoptotic bodies (AB), and cellular shrinkage (CS) were observed in WBR-treated HT-29 cells (Figure 1B-D).

Fluorescence analysis of mode of cell death

In order to examine whether the cytotoxic effects of WBR, as identified in our earlier study (Tan *et al.*, 2013) was due to apoptosis, the HT-29 cells were exposed with different concentrations (16, 32, 64 µg/mL) of WBR for 24 and 48 hours and stained with AO and PI fluorescence dye. As shown in Figures 2B-C, no distinct changes were observed in HT-29 cells after treatment with 16 and 32 µg/mL of WBR for 24 hours. Consistent with the changes observed in HT-29 cells after 24 hours incubation, a similar trend (no prominent changes) was observed in HT-29 cells after treated with 16 µg/mL for 48 hours (Figure 2B). Furthermore, HT-29 cells after treated with 64 µg/mL for 24 and 48 hours, respectively, and stained with AO and PI revealed chromatin condensation (CC) when observed using a fluorescence microscope (Figure 2D). A typical feature of apoptosis such as CC was also observed in the group of HT-29 cells treated with 32 µg/mL of WBR for 48 hours (Figure 2C). These apoptotic cells were observed in fluorescent bright-green color. However, the untreated HT-29 cells (control) demonstrated intact, round, and large green nuclei as shown in Figure 2A. Besides apoptotic cells, necrotic cells (red nucleus) were observed after 48 hours exposure with WBR (Figures 2B-D). None of the necrotic cells were observed in HT-29 cells after 24 hours exposure to WBR (Figure 2). The

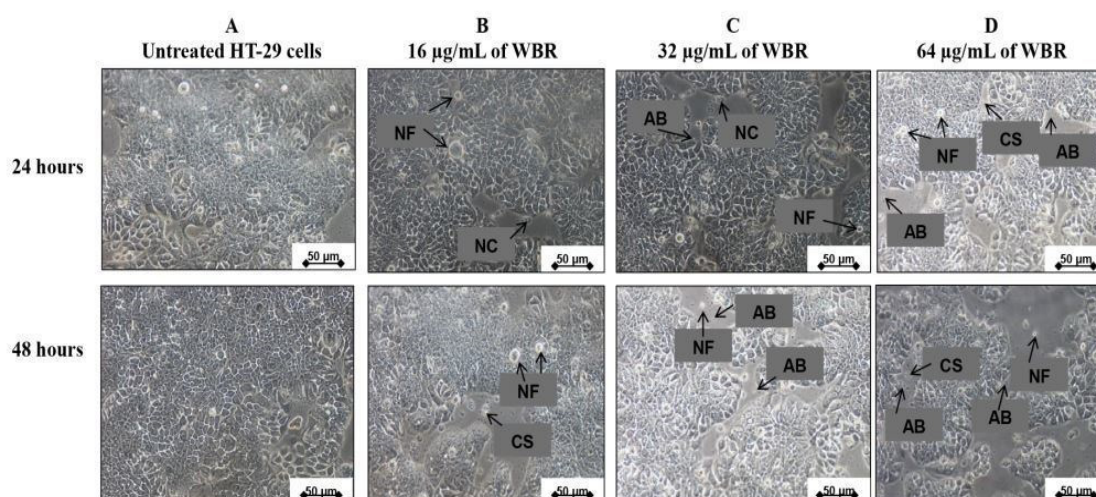


Figure 1. Morphological changes observed by inverted light microscope (200×). Human colorectal cancer (HT-29) cell line was either untreated (A) or was induced treatment with water extract of brewers' rice for 16 µg/mL (B), 32 µg/mL (C), and 64 µg/mL (D) for 24 and 48 hours. Apoptotic bodies (AB), cellular shrinkage (CS), nuclear compaction (NC), nuclear fragmentation (NF)

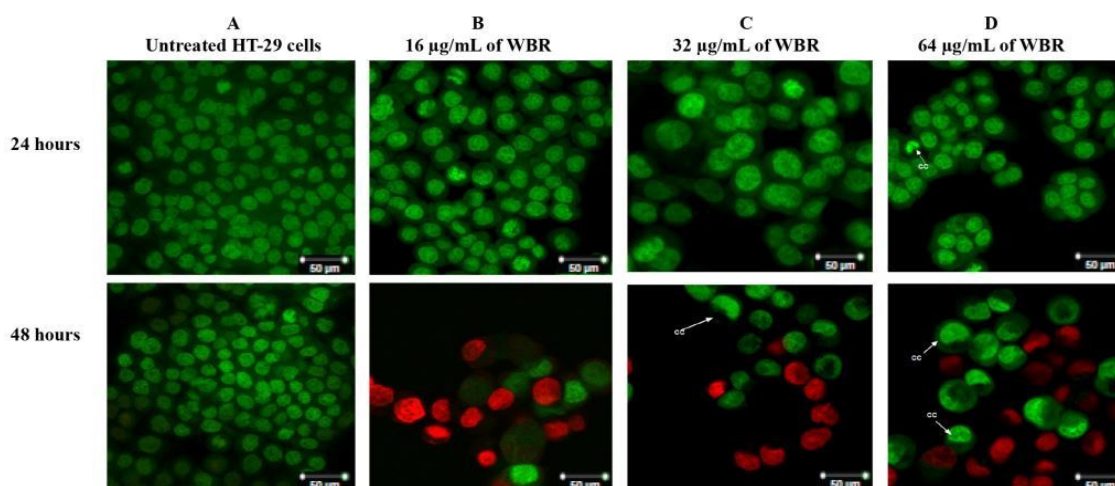


Figure 2. Morphological characterizations of cells undergo apoptotic death by acridine orange and propidium iodide staining (magnification 400×). Human colorectal cancer (HT-29) cell line was either untreated (A) or was induced treatment with water extract of brewers' rice for 16 µg/mL (B), 32 µg/mL (C), and 64 µg/mL (D) for 24 and 48 hours. Chromatin condensation (CC)

typical characteristic of apoptosis observed in AO and PI double staining in our study reconfirmed the apoptosis-inducing capability of WBR in HT-29 cell line as observed in an inverted light microscope.

Annexin V-FITC and PI staining apoptosis test

To ascertain whether WBR induced apoptosis in HT-29 cells, we further examined the HT-29 cells in response of WBR at different concentrations (16, 32, and 64 µg/mL) for 24 and 48 hours. As expected, the groups treated with 16, 32, and 64 µg/mL WBR for 24 hours significantly increased the number of early and late apoptotic HT-29 cells compared to untreated HT-29 cells ($p < 0.05$) (Figures 3(a) and 3(b)). Analysis of Annexin V-FITC and PI staining revealed that the groups treated with WBR resulted in a significant

increase in the total apoptotic cells, which can be observed after 24 hours, with a maximum effects observed in the group treated with 32 µg/mL of WBR ($p < 0.05$). The data presented in this study demonstrated a majority of the HT-29 cells treated with WBR exhibited total apoptotic cells compared to the necrotic counts (<5%).

Consistent with the apoptotic effects observed after 24 hours of treatment, the groups treated with 16, 32, 64 µg/mL of WBR also demonstrated a significant increase of early and late apoptotic cells compared to untreated HT-29 cells after 48 hours incubation periods ($p < 0.05$). Collectively, the total apoptotic cells were significantly increased after 48 hours exposure to WBR compared to the untreated HT-29 cells ($p < 0.05$). As shown in Figure 4,

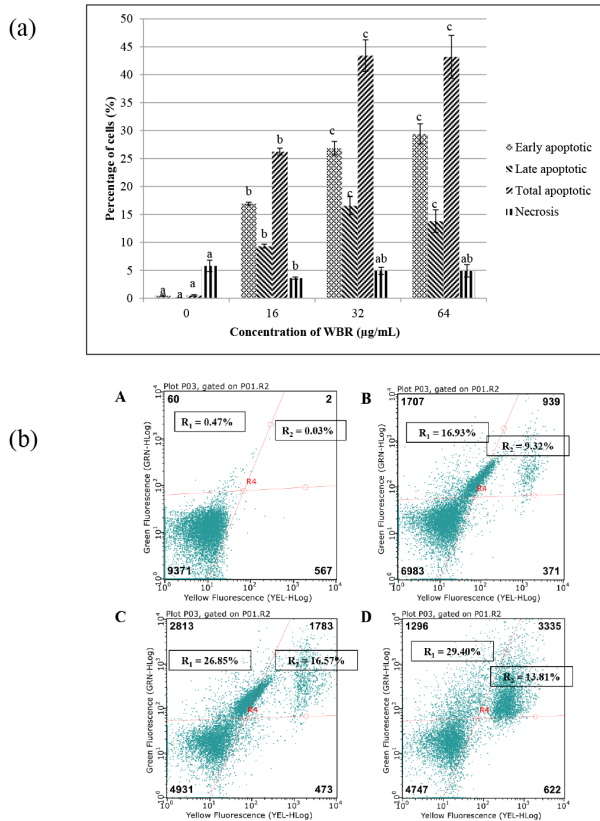


Figure 3. Apoptotic cell death of HT-29 cells treated with water extract of brewers' rice (WBR). After (a) 24 hours exposure to WBR, apoptosis was evaluated by Annexin V-FITC and propidium iodide staining assay. Values are reported in mean \pm standard deviation ($n = 3$), and values with different superscript letters demonstrate a significant difference as assessed using Tukey's test ($p < 0.05$). (b) Flow cytometric analysis of Annexin V-FITC and propidium iodide staining. Human colorectal cancer (HT-29) cell line was either untreated HT-29 cells (A), or was induced with WBR 16 µg/mL (B), 32 µg/mL (C) and 64 µg/mL (D) for 24 hours (R_1 = early apoptosis, R_2 = late apoptosis)

Annexin V-FITC and PI staining indicated that the majority of apoptotic cells were present in the groups treated with WBR compared to the untreated HT-29 cells. A very low necrotic count ($< 7\%$) was observed in the WBR treated groups. The necrotic counts in the group treated with 32 µg/mL of WBR was slightly higher than untreated HT-29 cells, but this difference was not significant ($p > 0.05$) (Figure 4). Collectively, these findings suggest that WBR may represent a promising natural dietary agent. Thus, our overall analyses indicated that WBR inhibited the proliferation of HT-29 cells via induction of apoptosis.

Cell cycle analysis by flow cytometer

Cell cycle plays a crucial role in the development,

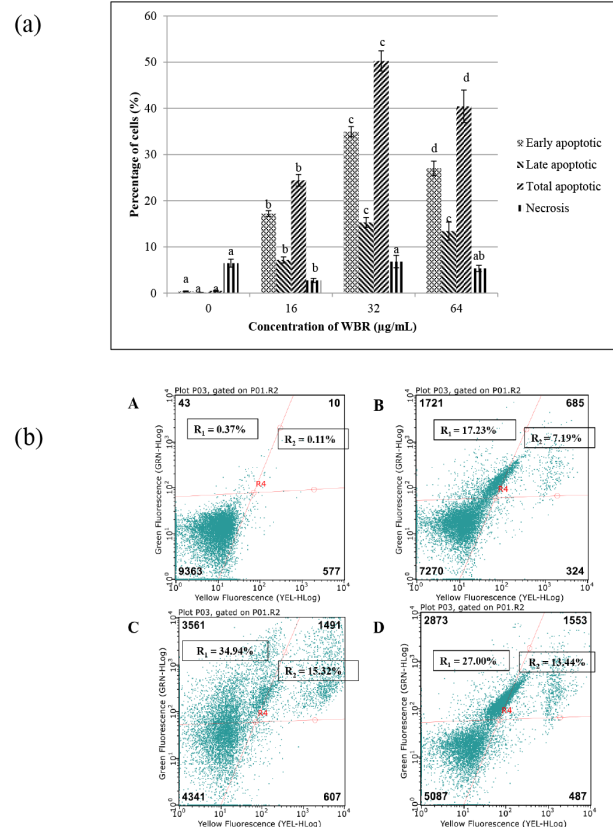


Figure 4. Apoptotic cell death of HT-29 cells treated with different doses of water extract of brewers' rice (WBR). After (a) 48 hours exposure to WBR, apoptosis was evaluated by Annexin V-FITC and propidium iodide staining assay. Values are reported in mean \pm standard deviation ($n = 3$), and values with different superscript letters demonstrate a significant difference as assessed using Tukey's test ($p < 0.05$). (b) Flow cytometric analysis of Annexin V-FITC and propidium iodide staining. Human colorectal cancer (HT-29) cell line was either untreated HT-29 cells (A), or was induced with WBR 16 µg/mL (B), 32 µg/mL (C) and 64 µg/mL (D) for 48 hours (R_1 = early apoptosis, R_2 = late apoptosis)

differentiation, and proliferation of mammalian cells (Schwartz and Shah, 2005). Because WBR displayed promising cytotoxicity capability and demonstrated to be a quite selective for HT-29 cells (Tan *et al.*, 2013), we next determined the regulation of cell cycle distribution in the presence of WBR in HT-29 cells by flow cytometry. In addition to the apoptotic effects observed via morphological evaluations as observed under inverted light microscope and fluorescence microscope and Annexin V-FITC and PI staining apoptosis test, we found that the changes in the cell cycle distribution demonstrated a dose-dependent accumulation of the HT-29 cells in the sub-G₀ phase after treatment with WBR for 48 hours (Figure 5). As shown in Figure 5, untreated HT-29 cells during the exponential growth period were characterized by

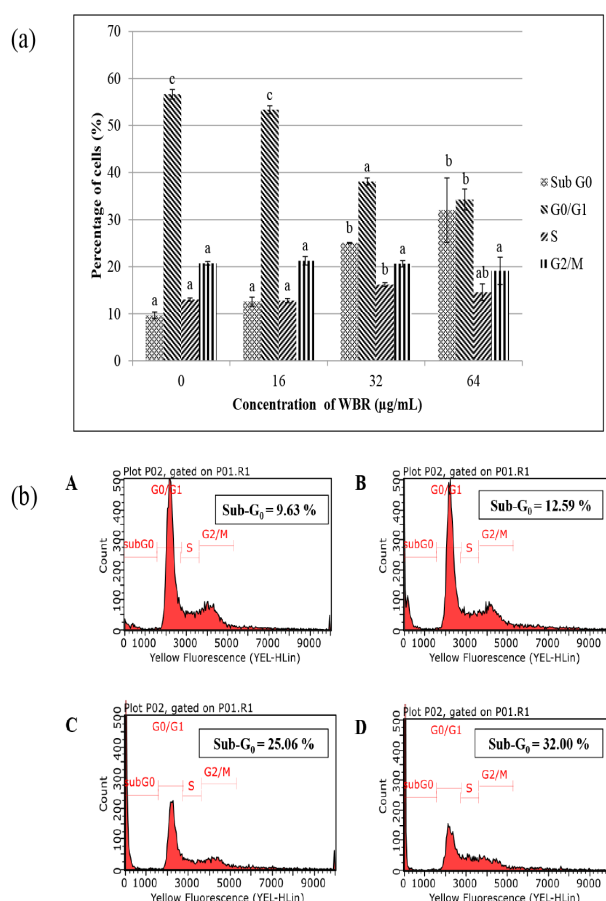


Figure 5. Cell cycle kinetics of water extract of brewers' rice (WBR) treated in HT-29 cells. (a) After 48 hours exposure to WBR, the cell cycle kinetics was analyzed using flow cytometry. Values are reported in mean \pm standard deviation ($n = 3$), and values with different superscript letters demonstrate a significant difference as assessed using Tukey's test ($p < 0.05$). (b) Flow cytometry analysis of cell cycle by RNase/PI staining. Colorectal cancer (HT-29) cell line was either untreated (A) or was induced by WBR 16 $\mu\text{g/mL}$ (B), 32 $\mu\text{g/mL}$ (C), and 64 $\mu\text{g/mL}$ (D) for 48 hours

cell populations in the sub- G_0 ($9.63 \pm 0.73\%$), G_0/G_1 ($56.64 \pm 1.01\%$), S ($13.01 \pm 0.35\%$), and G_2/M ($20.72 \pm 0.39\%$) phase of the cell cycle.

Cell cycle evaluation revealed that after 48 hours exposure to 32 and 64 $\mu\text{g/mL}$ of WBR, a significant increase in the cell population at the sub- G_0 phase compared to the control (untreated HT-29 cells), indicated cell death ($p < 0.05$) (Figures 5a and 5b). HT-29 cells accumulated at the sub- G_1 phase because of the cleavage of nuclear DNA into multiple fragments (Han and Park, 2009), which imply induced cell death through apoptosis (Ng *et al.*, 2011). Furthermore, cell cycle analysis also revealed that no significant difference in sub- G_0 phase was observed between the untreated HT-29 cells and the group treated with 16 $\mu\text{g/mL}$ of WBR ($p > 0.05$), whereas the groups of cells treated with 32 and 64 $\mu\text{g/mL}$ of

WBR significantly decreased G_0/G_1 phase compared to the untreated HT-29 cells ($p < 0.05$). In S phase, no significant difference was observed between untreated HT-29 cells and those groups treated with 16 $\mu\text{g/mL}$ or 64 $\mu\text{g/mL}$ of WBR ($p > 0.05$). A similar trend (no significant difference) was also observed in G_2/M phase between the untreated HT-29 cells and those groups treated with 16 $\mu\text{g/mL}$, 32 $\mu\text{g/mL}$, or 64 $\mu\text{g/mL}$ WBR ($p > 0.05$), without any cell cycle arrest.

Apoptosis is another approach used to destroy cancer cells (Shankar *et al.*, 2008). It acts as a protective mechanism to eliminate damaged cells prior to the manifestation of malignancy (Singh *et al.*, 2011) without the stimulation of inflammatory responses (Majno and Joris, 1995). Therefore, apoptosis induction is a crucial strategy in the cancer chemoprevention and chemotherapy (Khan *et al.*, 2007). In the present study, a plausible justification to explain this antiproliferation of HT-29 cells and subsequently contributed to the apoptotic cell death may be the presence of bioactive components found in brewers' rice such as phenolic antioxidants, phytic acid (Tan *et al.*, 2013), and dietary fiber (Tan *et al.*, 2014).

Biological activities in the cereal grains were strongly correlated to their polyphenol antioxidants (Awika *et al.*, 2003), which is well-recognized to exert numerous biological effects (Zieliński and Kozłowska, 2000; Heim *et al.*, 2012; Rastija and Medić-Šarić, 2009). The correlation between biological activities and the bioactive compounds further proved that the observed apoptotic effects seen in this study may be mediated partly via the synergistic/additive effects of these bioactive constituents. Furthermore, WBR did not cause any cytotoxic effect on normal cell line (BALB/c 3T3) as measured by MTT assay (Tan *et al.*, 2013), suggest that WBR is safe to be consumed.

Conclusions

Our data in the present study provide clear evidence that WBR inhibits the growth of human colorectal cancer (HT-29) cells via induction of apoptosis even after treated in a short duration (24 and 48 hours). Because brewers' rice is edible, thus it is potential to be used to process into nutritious food products such as noodles and breakfast cereal. Taken together, we suggest that WBR may be a potential candidate for the prevention and treatment of colorectal cancer.

Acknowledgments

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